Regulation of Antioxidant Enzymes in Lung after Oxidant Injury

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Studies have implicated active oxygen species (AOS) in the pathogenesis of various lung diseases. Many chemical and physical agents in the environment are potent generators of AOS, including ozone, hyperoxia, mineral dusts, paraquat, etc. These agents produce AOS by different mechanisms, but frequently the lung is the primary target of toxicity, and exposure results in damage to lung tissue to varying degrees. The lung has developed defenses to AOS-mediated damage, which include antioxidant enzymes, the superoxide dismutases [copper-zinc (CuZnSOD) and manganese-containing (MnSOD)], catalase, and glutathione peroxidase (GPX). In this review, antioxidant defenses to environmental stresses in the lung as well as in isolated pulmonary cells following exposure to a number of different oxidants, are summarized. Each oxidant appears to induce a different pattern of antioxidant enzyme response in the lung, although some common trends, i.e., induction of MnSOD following oxidants inducing inflammation or pulmonary fibrosis, in responses to oxidants occur. Responses may vary between the different cell types in the lung as a function of cell-cycle or other factors. Increases in MnSOD mRNA or immunoreactive protein in response to certain oxidants may serve as a biomarker of AOS-mediated damage in the lung. — Environ Health Perspect 102(Suppl 2):79–87 (1994).

Key words: antioxidant enzymes, oxidants, regulation, ozone, hyperoxia, mineral dust, paraquat, smoke, bleomycin

Introduction

Many recent research efforts have highlighted the role of active oxygen species (AOS) in the pathogenesis of various lung diseases including lung cancers, pulmonary fibrosis, adult respiratory distress syndrome, emphysema, chronic bronchitis, and pleural disease. AOS can be generated by a diverse group of exogenous physical and chemical agents such as ozone, hyperoxia, paraquat, nitrogen and sulfur oxides, mineral dusts, ultraviolet radiation, tumor initiators and promoters, and endotoxins. Alternatively, production of AOS can be facilitated by endogenous mechanisms involving specific cytokines such as tumor necrosis factor (TNF) and interleukin-1 (IL-1), or after respiratory burst by activated macrophages and neutrophils. AOS are produced intracellularly by many pathways including the mitochondrial electron transport system and other enzymes. These include cytochrome P_{450} enzymes, conversion of xanthine dehydrogenase to xanthine oxidase which reacts with xanthine to produce superoxide anion ($\bullet O_2^-$), and NADPH oxidase, a membrane-associated enzyme in phagocytes. The most commonly recognized AOS are $\bullet O_2^-$, hydroxyl radical ($\bullet OH$), and hydrogen peroxide (H_2O_2). However, a wide variety of other metabolites of reduced oxygen exist which may be generated by oxidation of arginine to peroxynitrite, during metabolism of nitric oxides which may react with $\bullet O_2^-$ at diffusion limited rates (*1–4*), or by the reactions between H_2O_2 and myeloperoxidase to form hypochlorous acid (*5*).

The last decade of research has illuminated the importance of exogenous and endogenous antioxidants in lung defense including cellular enzymes which detoxify AOS. These enzymes, known collectively as the antioxidant enzymes, appear to be differentially regulated according to the type of oxidant injury inflicted. Alternatively, certain stresses may induce selective expression of one type of antioxidant enzyme far more than the others. The objective of this review is to describe regulation of various antioxidant enzymes after different types of oxidant stresses to the lung. This information is important in designing possible effective strategies for prevention of or therapeutic intervention in environmental lung diseases.

Pathways of Active Oxygen Species Generation

The initial step in the formation of AOS involves the generation of $\bullet O_2^-$ by a single electron donation to molecular oxygen (O_2), resulting in a very reactive unpaired electron state. This process can occur intracellularly in a nonenzymatic manner during the passage of electrons through the mitochondrial electron transport system. Alternatively, $\bullet O_2^-$ can be produced enzymatically by various cellular oxidases (xanthine oxidase, NADPH oxidase, etc.). H_2O_2 is formed when two molecules of $\bullet O_2^-$ dismutate either enzymatically or spontaneously to produce dioxygen and H_2O_2. In this way, generation of $\bullet O_2^-$ almost invariably leads to H_2O_2 production; however, a two-electron reduction of O_2 catalyzed by oxidases can lead directly to H_2O_2, without previous formation of $\bullet O_2^-$. Adding one electron to the oxygen-oxygen bond of H_2O_2 results in cleavage of the bond and subsequent release of a hydroxide anion and the very reactive species, $\bullet OH$. This electron addition is catalyzed by transition metal cations, such as ferrous iron (Fe^{+II}), in which the iron is converted from the ferrous to the ferric (Fe^{+III}) state, in a process known as the Haber-Weiss, or modified Fenton reaction (*6*). Figure 1 illustrates these pathways of AOS formation and the antioxidant enzymes involved in their detoxification.

Antioxidant Enzymes

The antioxidant enzyme "family" includes catalase, glutathione peroxidase (GPX), and the manganese and copper-zinc superoxide dismutases. Other enzymes newly recognized as responsive to oxidant stress, (heme oxygenase; metallothionein, heat shock proteins) are not exclusively regarded as antioxidants and will not be reviewed here. Frequently these enzymes act in con-

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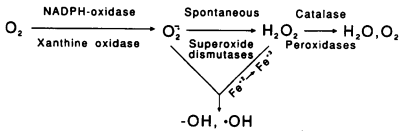

Figure 1. Generation of active oxygen species (AOS) and antioxidant enzymes involved in their catabolism.

cert to protect lung or other tissue from oxidant damage. The superoxide dismutases, which are the product of highly conserved genes, protect cells by converting $\bullet O_2^-$ to H_2O_2. This molecule, which is in itself toxic to cells, can, as mentioned previously,be broken down to release hydroxyl radical (•OH), a reactive species far more damaging to cells than either $\bullet O_2^-$ or H_2O_2. The enzymes responsible for detoxifying H_2O_2, namely catalase and GPX, prevent the formation of •OH by converting H_2O_2 to other less harmful products. Thus, this enzyme family may act in a sequential fashion to "dismutate" one toxic active oxygen species to another, which then can be rapidly broken down to nontoxic byproducts (Figure 1).

The Dismutases

Copper-zinc superoxide dismutase (CuZnSOD) is a dimer containing copper for its catalytic action and zinc as a protein stabilizer. This enzyme may be localized in the cytoplasm and nuclear matrix (*6–8*) or in peroxisomes (*9*). CuZnSOD reduces $\bullet O_2^-$ in the reaction:

$$SOD\text{–}Cu^{2+} + \bullet O_2^- \rightarrow SOD\text{–}Cu^{1+} + O_2$$

$$SOD\text{–}Cu^{1+} + \bullet O_2^- + 2H^+ \rightarrow SOD\text{–}Cu^{2+} + H_2O_2$$

Manganese superoxide dismutase (MnSOD), the mitochondrial form of the dismutases, is a protein tetramer, contains manganese for its catalytic activity and reduces $\bullet O_2^-$. MnSOD is compartmentalized in the mitochondria presumably to detoxify $\bullet O_2^-$ generated by the electron transport system (*6*).

Two other forms of superoxide dismutases also exist. Iron superoxide dismutase (Fe–SOD) is primarily a procaryotic SOD (but is also found in several plant families) and contains iron in its catalytic site. Fe–SOD is more closely related to MnSOD than CuZnSOD, based on amino acid sequence homology and X-ray structure, and may coexist with Mn–SOD in some bacteria (*10,11*). Extracellular superoxide dismutase (EC–SOD) is the predominant SOD found in the extracellular or interstitial space, contains copper and zinc, and has affinity for heparan sulfate and proteoglycans located on endothelial and other cell surfaces (*12,13*).

Catalase and Glutathione Peroxidase (GPX)

Catalase, a 240,000 molecular weight protein tetramer, is located primarily in cytosolic peroxisomes. It functions to detoxify H_2O_2 to oxygen and water in the reaction:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Catalase substrates generally consist of small molecules including H_2O_2 and the methyl or ethyl hydroperoxides. Catalase exhibits high reaction capacity, but has a high K_m value (10^7/M·sec) for H_2O_2 (*6*).

Glutathione peroxidase, also a tetramer, is an 85,000 dalton protein containing selenium, and uses glutathione as a co-substrate. GPX is a cytosolic enzyme and also eliminates H_2O_2; but, in comparison to catalase, has a wider range of substrates including lipid peroxides. The kinetics of this enzyme are very complex, but it is thought to have a greater affinity for H_2O_2 than catalase. Glutathione peroxidase primarily functions to detoxify low levels of H_2O_2 in the cell (*6*).

Antioxidant enzymes may act in a coordinate manner to defend living tissue from oxidant damage. As outlined above, two of the enzymes catabolize H_2O_2 and other peroxides, and two enzymes detoxify $\bullet O_2^-$. Since the enzymes which reduce $\bullet O_2^-$ are inactivated by H_2O_2, and the enzymes catabolizing H_2O_2 are inactivated by $\bullet O_2^-$, one enzyme system complements the other by detoxifying its corresponding inactivating oxidant. And, as a further benefit, the catabolism of H_2O_2 by GPX and catalase inhibits the formation of •OH. This defensive interplay may be critical for cell survival under oxidant stress.

Genes Encoding Antioxidant Enzymes

The human gene for CuZnSOD (*14*) is located on chromosome 21 and is present as single copy per haploid genome spanning 11 kb of chromosomal DNA. Structural analysis of this gene reveals five exons and four introns with an unusual variant dinucleotide sequence at the first intron (G–C instead of the highly conserved G–T). The 5' end of the gene contains both TATAA and CAT promotor sequences. Transcription of this gene results in two mRNA species, one of which is 200 bp longer than the other (0.9 and 0.7 kb, respectively), with the 0.7 kb species expressed approximately four times as often as the larger species.

The MnSOD gene in the rat, as determined by Ho and colleagues (*15*) consists of two genes per haploid rat genome. MnSOD I contain at least five exons and four introns, which results in five distinct mRNA species from 4 kb to 1 kb in length in the lung due to splicing. MnSOD I is located in one piece of a 16.4 kb EcoRI genomic DNA fragment. The MnSOD promotor is very complex with highly GC rich regions and multiple enhancer regions, an AP-1 binding motif, but no CAAT or TATAA box. This gene has been shown to be expressed under normal physiological conditions and is induced by oxidant stress. The MnSOD II gene structure is under further investigation at this time. Studies by Nick and associates (*16*) indicate the presence of only one MnSOD gene, with the multiple mRNA species attributed to alternate cleavage and polyadenylation.

The human catalase gene (*17*) has been mapped to chromosome 11, band p13. It is 34 kb long and consists of 13 exons and 12 introns. The introns range from 400 bp to 10.5 kb in length, with the largest separating exons 1 and 2. The catalase gene, like MnSOD, apparently lacks an upstream TATAA box, but does contain several GCAAT and GGGCGG regions which may form part of a promotor. Transcription of the catalase gene results in a single mRNA species 2.4 kb long.

The gene for GPX (*18*) has been mapped to chromosomes 3, 21, and X in human-rodent hybrid cell DNA. The gene on chromosome 3 contains 4 restriction sites and a single intron; in contrast, the genes on chromosomes 21 and X contain no intron. Great sequence homology exists between the genes on chromosomes 3 and X, with 21 exhibiting more divergence. Despite the homology between sites 3 and X, it is thought that only the gene on chromosome 3 is functional, with the two other sites containing processed pseudogenes.

Alterations in Antioxidant Enzymes after Exposure to Agents Inducing Oxidative Stress

Ozone

Ozone (O_3) is a highly reactive molecule forming naturally in the stratosphere where it acts as a shield to protect the Earth's surface from harmful levels of ultra-violet light (Figure 2). However, ozone also is created at the terrestrial level by the interaction of

oxygen, volatile organic compounds such as vapor phase hydrocarbons emitted from the burning of fossil fuels, NO_2, and sunlight in a photochemical reaction which produces the characteristic "smog" of our urban areas (*19*).

Ozone exerts its toxic effects in vivo primarily by oxidization. Targets include soluble components of alveolar lining fluid, lipids in cell membranes, and functional groups of enzymes and other biomolecules (including DNA) resident in the cell (*19–21*). A host of cellular functions are altered, and a cascade of events leading to acute inflammation has been demonstrated. Ozone manifests these actions by production of free radicals such as H_2O_2 and •OH, the formation of aldehydes and lipid peroxides when ozone interacts with lipid membranes, or in limited cases by direct interaction after diffusion through membranes (*19–23*).

The lung is the major organ affected by ozone toxicity, and ozone concentrations of 4 to 10 ppm are fatal in some animal studies (*19*). Sublethal toxicity has been definitively demonstrated at much lower levels (0.08–0.12 ppm) that approximate both common ambient conditions and the current national standard (*24,25*). In the upper airway, ozone is particularly damaging to the ciliated cells of the respiratory tract, whereas toxicity in the lower airway is most dramatic in the regions of the lung surrounding the terminal bronchiole/alveolar duct junction. In the alveolar region, destruction of type I alveolar cells occurs with type II alveolar cells being considerably more resistant to ozone toxicity (*26*). The degree of damage to lung tissue inflicted by ozone is not easily predicted from a simple concentration × time = dose model. Concentrations of ozone, exposure pattern, and host defenses (enzymatic and otherwise) clearly impact on ultimate toxicity (*19,27*).

Studies in the last several years have indicated that antioxidant enzymes play a role in the defense of mammalian lung tissue from damage incurred by exposure to ozone. Studies employing *in vitro* ozone exposure systems have generally not examined antioxidant enzyme gene expression, activity, or protein as end points for cell defense. Morgan (*28*) reported cell culture data implicating a protective role for catalase and SOD, as well as an oxidant scavenger (mannitol) in ozone related cytotoxicity. Acute *in vivo* exposure to ozone does, for example, cause initial glutathione (GSH) depletion in lung tissue, whereas continual exposures result in restoration of GSH levels within days. This increase correlates with increases in GPX, GSH-reductase, and G6PD activity and is apparent even with low-level exposures approximating common ambient conditions (*19,29,33*).

Increased activity of antioxidant enzymes occurs after several days of exposure to ozone (*19*). Recently, Rahman and colleagues (*31*) measured antioxidant enzyme mRNA and enzyme activity in whole lungs of rats exposed *in vivo* to 0.7 ppm ozone for 5 days. Measurable differences in gene expression were apparent at day 3 of exposure for both young and mature animals. The maximum response (2-fold increase) was detected for CuZnSOD with more subtle rises in glutathione peroxidase and catalase. MnSOD mRNA was not probed. Enzyme activity showed more modest increases, also varying with the time point and specific enzyme studied Another study conducted by the same group examined the antioxidant enzyme response to ozone exposure in heart and brain tissue. Rats exposed to 0.25 and 0.7 ppm ozone for 5 days showed increases in thiobarbituric acid-reactive material (an indicator of lipid peroxidation), and glutathione peroxidase and catalase activities in both brain tissue and the left ventricle of the heart (*32*). Since penetration of ozone may be limited to one membrane thickness, diffusion of more stable secondary reaction products including lipid peroxides may be important in injury to distal organs (*32,33*). These findings may implicate ozone concentrations now found in some urban areas in the pathogenesis of some forms of heart and brain disease (*29*).

Rats pretreated with endotoxin (*34*) to elevate MnSOD activity were virtually completely protected from lung edema due to ozone. These animals exhibited decreased lipid peroxidation and less loss of weight when compared to sham rats. This negation of ozone toxicity was accomplished without a corresponding increase in any other antioxidant enzyme activity.

Experiments using Japanese quail have revealed possible differences in defensive response to ozone exposure in avian versus mammalian lungs. Quail exposed to ozone concentrations ranging from 0 (control) to 3.0 mg/m^3 for 7 days showed no increase in antioxidant enzyme activities at the middle exposure levels (0.3 and 1.0 mg/m^3), and glutathione peroxidase levels were decreased at the high exposure level (3.0 mg/m^3 air) when corrected for increased lung weight (*35*). This lack of induction of

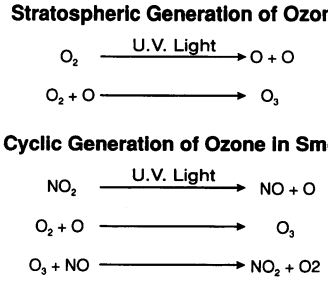

Figure 2. Schematic diagram indicating mechanisms of ozone generation. (Adapted from Mustafa (*19*).

antioxidant enzymes (and increased damage to the lung when compared to mammalian species) may be due to an absence of epithelial cell proliferation in response to ozone as avian lung is devoid of any cell corresponding to the mammalian type II epithelial cell. Since this cell is involved in repair of the alveolar lining after oxidant damage in mammals, lack of this cell type in avian lung may render them more susceptible to ozone-inflicted injury (*35*). It should be noted that substantial differences between mammalian species exist in ability to respond to ozone as demonstrated by antioxidant enzyme induction using either whole lung assay or microdissection (*35,36*). Comparisons of antioxidant enzyme activity in rat and monkey lung tissues exhibited variability between lung subcompartments within and across species, and differences were found in enzyme activities in whole lung homogenates between species. These differences may impact on the ability of the two species to cope with ozone exposure (*36*).

The role of the traditional antioxidant enzymes in protecting the lung from ozone is presumed, but unproven. They clearly increase, albeit in variable quantities, at mRNA, protein, and activity levels with ozone exposure, but whether these increases provide clear evidence of protection is unknown. Other experimental strategies, such as the execution of *in vitro* gene transfection studies and *in vivo* exposure of transgenic animals, may be helpful in resolving this question.

Hyperoxia

Hyperoxia-induced lung damage is of great clinical interest due to the use of oxygen therapy in the care and management of many hospital patients. Oxygen therapy has been implicated in the development of adult respiratory distress syndrome and bronchopulmonary dysplasia in the new-

born (*37*). Adult rats exposed to 100% oxygen exhibit respiratory distress at 48 hr and die within 72 hr after exposure is initiated (*38*). Specific targets of hyperoxic insult to the lung appear to be the epithelial cells of the alveoli and the vascular endothelial cells (*38,39*). Hyperoxia damages these cells by the generation of AOS, and, like ozone, injures type I alveolar epithelial cells primarily with type II cells being much more resistant. A compensatory mechanism exists in the lung in response to hyperoxic damage—after type I cells die, type II alveolar epithelial cells rapidly divide to repopulate the lung and can differentiate into type I cells (*6,37,38*). Hyperplasia also occurs in interstitial cells of the lung which, when combined with existing proliferation of alveolar epithelial cells, inhibits the ability of the lung to perform its gas exchange function. Pre-exposing rats to hypoxic conditions significantly reduces the amount of tissue hyperplasia when they are subsequently subjected to high oxygen levels (*38*).

Whether the adaptation of type II alveolar epithelial cells to hyperoxic conditions is the result of increased production of antioxidant enzymes in these cell types when stressed by exposure to AOS has been explored (*37*). Rats exposed to concentrations of 85% or more concentrations of O_2 show increased activities of some antioxidant enzymes in isolated type II cells when compared to sham, air-exposed rats. In one study, CuZn and MnSOD showed heightened activities (43 and 28%, respectively) after normalizing data to cytochrome c levels. The authors conclude that hyperoxia causes true increases in activities of the superoxide dismutases in whole lung, whereas observed increases in activity of the other AOEs may be due to hypertrophy of the type II cells (*37*).

In rat neonatal lung, the increased activity of CuZn SOD is tied to an increase in mRNA for this enzyme, demonstrating that control exists at the transcriptional level. In adult rats, transient hyperoxic conditions result in increased synthesis of CuZnSOD without corresponding increases in mRNA levels or activity of the enzyme (*40*). Apparently, at some point between the late gestational period and adulthood, there is a switch from transcriptional to translational control of synthesis of this enzyme. Adult rats exposed to >95% O_2, allowed a brief rest period, and re-exposed to hyperoxic conditions, also exhibit increased CuZnSOD activity along with increases in mRNA, indicating a return to transcriptional regulation of enzyme synthesis (*40*). MnSOD exhibits increased expression of mRNA in response to hyperoxic stress, with levels sometimes reaching 5- to 10-fold. The heightened mRNA expression does not result in increased activity of the enzyme until some 7 to 10 days of exposure even though the mRNA is increased much earlier (*41*). An earlier study by Crapo and Tierney found increases in lung SOD activity as early as 5 days of exposure (*42*). This indicates that both transcriptional activation and translational/post-translational control of this enzyme occur in response to oxidant stress (*41*).

Rat airway epithelial cells may be resistant to hyperoxia by possessing constitutively higher levels of the antioxidant enzymes than other lung parenchymal cells. In an experiment in which rats were exposed to 85% O_2, sham control rats showed high levels of immunolabeling for both superoxide dismutases and catalase in epithelial cells of all airways, with the cells of the larger airways having the highest basal levels (*43*). The ciliated cells of these airways exhibited higher levels of CuZnSOD and MnSOD than secretory cells. After exposure to hyperoxic conditions, no increases were found in any of the cell types examined. Few cellular morphological changes were found in the bronchi or bronchioles, and no significant inflammatory response was found in any area of the airway, indicating that the increased resistance of airway epithelial cells to hyperoxic stress may be mediated by the higher basal levels of antioxidant enzymes contained in these cells (*43*). Instillation of red blood cells into the tracheobronchial tree of rats dramatically prolonged their survival in hyperoxic conditions, with the protective effects being primarily attributable to the glutathione redox system, of which GPX is a constituent (*44*).

Further evidence for the protective role of antioxidant enzymes in lung tissue is provided by studies using mice transfected with the human MnSOD gene (*45*). These mice showed 40% increases in MnSOD activity in whole lung homogenates compared to transgene negative mice. Increased immunoreactive protein was localized in mitochondria of type II alveolar cells by immunogold labeling techniques, and was not found in fibroblasts, type I cells, or epithelial cells, while expression of other antioxidant enzymes were not elevated. Transgenic mice (6 to 8 weeks old) exposed to 96% oxygen showed striking increases in survivability over nontransgenic mice (from 125 ± 18 hr for controls to 200 ± 12 hr for transgenic mice). Increased survivability was also noted in 5- to 6-month-old transgene-positive mice exposed to the same oxygen levels (*45*). Young transgenic mice (2.5 months old) with constitutively high levels of CuZnSOD evidenced increased survivability when exposed to >99% O_2 in comparison to control mice, while older transgenic mice (5.5 months) exhibited no increase in survival. Continuous exposure to hyperoxia resulted in increased activity of glutathione peroxidase and catalase and a decline in SOD (*46*).

Cultured endothelial cells also reveal increases in antioxidant enzyme activities when exposed to hyperoxic conditions. Exposures of endothelial cells to 95% O_2 for 3 days results in elevated mRNA levels and enzyme activities of CuZnSOD and glutathione peroxidase, an increase in mRNA for catalase, but a decrease in activity and no change in mRNA levels or activity of MnSOD. At 5 days, mRNA levels for all antioxidant enzymes were increased, and the activities of GPX and CuZnSOD were elevated. Catalase activity decreased even further, and MnSOD remained unchanged. Glutathione peroxidase showed the highest increase in both activity and mRNA expression indicating that in this cell type, glutathione peroxidase is the major enzyme of importance in the detoxification of hydrogen peroxide (*47*).

Regulation of expression of antioxidant enzymes can also be affected by cytokines. For example TNF and IL-1, when administered to rats before exposure to hyperoxic conditions, play a role in reducing the damage caused by increased O_2 tension (*48,49*). TNF has two protein forms, alpha and beta, both secreted by mononuclear cells. Alpha is produced primarily by macrophages, and beta by lymphocytes, while IL-1 is functionally related to the TNFs and is produced by macrophages. All exert their protective effects by increasing the expression of MnSOD mRNA and enzyme levels. The other antioxidant enzymes are not affected by these cytokines (*48,49*).

Under hyperoxic conditions, alveolar macrophages exhibit increased production of TNF *in vitro*. After isolation of alveolar macrophages from rats and subsequent incubation with lipopolysaccharide (LPS), a known stimulator of TNF production, statistically significant increases in TNF production were found in macrophages exposed to 95% O_2 and treated with LPS concentrations of 0 and 0.001 μg/ml (*50*). This study indicates that hyperoxia is a

potent source of elevated TNF production in macrophages and may elucidate one of the mechanisms by which hyperoxic conditions stimulate increases in MnSOD levels in rat alveolar macrophages.

Mineral Dusts (Asbestos and Silica)

Asbestos refers to a diverse family of crystalline hydrated fibrous silicates with a greater than 3:1 length to diameter ratio. Asbestos has two major classes: serpentine (of which chrysotile is the only type), and amphibole. Crocidolite, amosite, anthophyllite, tremolite, and actinolite are included in the amphibole class. Chrysotile consists of curly, pliable fibers that tend to bundle together. In contrast, members of the amphiboles class have straight, needle-like fibers (*51,52*). Chrysotile accounts for over 90% of the world's asbestos production, with some amphiboles, particularly crocidolite, accounting for the remainder (*52*). Silica also exists as many polymorphs including alpha quartz, min-U-sil, amorphous silica, and alpha cristobalite (*53*).

Asbestos exposure has been implicated in the development of at least three types of lung disease: asbestosis, lung cancer, and malignant mesothelioma. The features of asbestos-associated diseases have been described previously (*51,52*). Silica exposure is implicated in the development of silicosis, a disease dependent upon intense protracted exposure to silica, usually in the workplace (*54*). Silicosis, like asbestosis, is characterized by inflammation in the lung, with collections of alveolar macrophages and reticulin fibers forming the initial lesion. In time, the classic silicotic nodule is formed. Short, intense exposure to silica may lead to the acute form of silicosis, termed silica-proteinosis, which is characterized by extensive damage to airway epithelial cells, type II cell hyperplasia, and a protein and lipid-rich exudate in the air spaces (*54*).

One likely causative factor in mineral dust related lung injury is the formation of AOS by redox reactions catalyzed by minerals (*55,56*). For example, in cell free systems, iron present on the surface of both asbestos fibers and silica particles can drive a modified Haber-Weiss reaction which converts superoxide anion and hydrogen peroxide to the hydroxyl radical (*57–59*). The greater the iron content, the more AOS generated. In the case of silica, greater oxidant generation occurs when it is freshly crushed (*60,61*). Cells exposed to crocidolite pretreated with deferoxamine (an iron chelator) suffered substantially less cytotoxicity than cells exposed to untreated crocidolite, highlighting the role of iron in AOS generation (*62,63*). Treatment of silica with iron chelators also diminishes its oxidant generating capability (*59*).

Asbestos-mediated AOS generation also occurs when inhaled fibers are phagocytosed by alveolar macrophages or other cell types (*63,64*). If the fiber is long, the cell cannot completely ingest it, a phenomenon known as frustrated phagocytosis (Figure 3). The resultant respiratory burst then generates AOS which can leak out into the external environment of the lung damaging surrounding cells (*52,64*). Rat alveolar macrophages and human neutrophils generated substantial AOS when exposed to silica (*60*) or asbestos (*64*), as determined by electron spin resonance and cytochrome c reduction. Addition of catalase to the incubation media more effectively inhibited AOS generation than did addition of SOD, implicating H_2O_2 and •OH as the major AOS produced by phagocytosis of these mineral dusts (*59*).

The elaboration of TNF by alveolar macrophages and other inflammatory cells may also be involved in the generation of AOS in the lung during mineral dust exposure. The cytotoxic effects of TNF are thought to be mediated partly by the production of AOS, including hydrogen peroxide and $\bullet O_2^-$ when TNF interacts with the target cells membranes or other components such as mitochondria (*65–67*).

Production of AOS after inhalation of asbestos or cristobalite silica has been linked to both increases in gene expression and antioxidant enzyme activity in lung tissue. Rats exposed to crocidolite (*7–10* mg/m^3 air) show increases in the activity of all measured antioxidant enzymes in lung tissue, with slight differences in the extent and time frame of increases (*68–70*). These increases in enzyme activity correlate with increased mRNA expression for enzymes, with the exception being CuZnSOD which showed no statistically significant increase over control rats. MnSOD exhibits most dramatic increases in steady-state mRNA levels which peak at 6 to 9 days and then decline to near normal levels at 14 days postexposure. Glutathione peroxidase and catalase exhibited increases in mRNA levels at various time points, but not as high or as consistently elevated as MnSOD (*68–70*).

Related studies with cristobalite silica at similar dosages have elucidated somewhat different patterns of antioxidant enzyme expression (*68*). The greatest increase in mRNA induction occurred with MnSOD, with higher levels found than those previously observed after exposure to similar concentrations of asbestos with MnSOD mRNA remained elevated at 14 days postcessation of exposure to silica. In general, the other antioxidant enzymes (catalase, GPX) exhibited no striking changes in mRNA levels, and CuZnSOD was unaffected by exposure to silica. Increases in

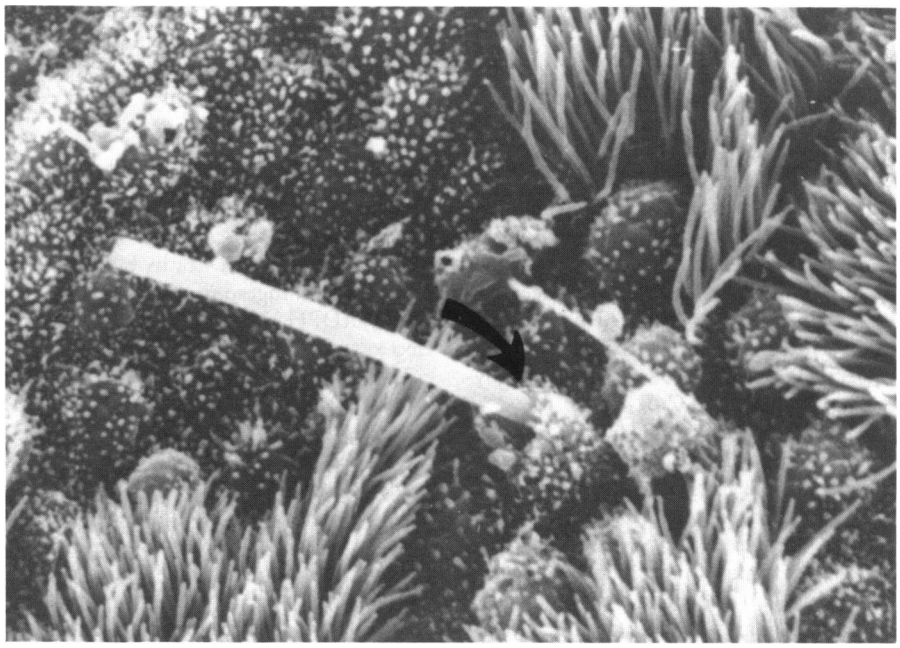

Figure 3. Scanning electron micrograph of interaction between an incompletely phagocytized asbestos fiber (arrow) and a tracheal epithelial cell in organ culture. The illustrated phagocytosis of minerals may cause generation of active oxygen species (AOS).

MnSOD mRNA correlated with increased immunoreactive protein levels; however, no increases in enzyme activities of antioxidant enzymes were observed (*68*). Increases in MnSOD immunoreactive protein were attributed to increased induction per mitochondrial area in type II epithelial cells and correlated directly with the degree of inflammation and pulmonary fibrosis observed in animal models of silicosis and asbestosis (*71*).

The disparity between the superoxide dismutases in terms of selective mRNA induction in lung is interesting. Although the two enzymes are compartmentalized in the cell (MnSOD in mitochondria and CuZnSOD in cytoplasm and peroxisomes) both forms are encoded in the nucleus. Apparently the two enzymes are not regulated coordinately when cells of the lung are exposed to asbestos, silica or chemical generating systems of AOS (*68,72*).

These conclusions are supported by studies which indicate the expression of these genes is differentially regulated when cells are exposed to TNF and IL-1. Both of these cytokines induce increased mRNA levels for MnSOD but not for CuZnSOD (*48,49,65*). Increased TNF mRNA expression has also been found in rat lungs exposed to crocidolite asbestos or cristobalite silica (*73*), and after intratracheal administration of silica (*74*). However, it is not entirely clear whether TNF induces antioxidant enzyme gene expression by direct interaction with these genes, or by another mechanism such as production of AOS.

Recent experiments using in vitro cultures of human mesothelial cells demonstrated 2- to 3-fold increases in steady-state MnSOD mRNA levels when these cells were exposed to either crocidolite or chrysotile asbestos or to xanthine and xanthine oxidase (*75*). These data support the conclusions that the response of antioxidant enzymes to mineral dust-induced cellular damage is controlled at the transcriptional level with independent molecular regulation of mechanisms governing MnSOD and CuZnSOD.

There is evidence that not all lung cell types express comparable increases in antioxidant enzymes after stress. This may account for the selective focal cell damage seen in certain types of oxidant stress. Recent experiments have focused on localizing MnSOD content in the various lung cells, using immunocytochemical techniques (*71*). In rats exposed to crocidolite or cristobalite, MnSOD protein is primarily expressed in the mitochondria of type II alveolar cells, with lung fibroblasts containing very little and type I alveolar cells and endothelial cells containing no detectable immunoreactive protein. The increases in MnSOD in type II epithelial cells in response to mineral dust correlated with the 2-fold or greater increases in immunoreactive protein found in whole lung homogenates (*71*). This finding is in agreement with the concept that type II alveolar cells may be resistent to certain types of oxidant stress because of induction of MnSOD or other antioxidant enzymes.

It appears doubtful that increases in antioxidant enzymes in response to mineral dusts at high concentrations are sufficient enough to protect the lung from oxidant damage. For example, when polyethylene-glycol conjugated catalase was administered to rats during exposure to asbestos, the enzyme-treated rats suffered reduced damage to lung tissue in comparison to untreated rats. However, the dose of catalase needed to inhibit this damage was much greater than the approximately 2-fold increases in antioxidant enzymes observed in lungs of rats after exposure to asbestos (*76*). The long lag time before induction of antioxidant enzymes after mineral dust exposure (6 days for catalase, even longer for the other antioxidant enzymes) and the lack of marked induction until after irreversible damage has occurred, indicates that lung defense mechanisms at high concentrations of agents are insufficient. Lastly, the fact that antioxidant enzymes are not uniformly distributed throughout the cells of the lung implies that some cell types may be relatively unprotected (*71*).

Paraquat

The herbicide paraquat (1,1-dimethyl-4,4-bipyridylium dichloride) has been implicated in hundreds of deaths by accidental or intentional ingestion. Paraquat interacts with NADPH resulting in a cyclic single electron redox reaction of the parent molecule generating the paraquat radical, which in the presence of molecular oxygen, releases $\bullet O_2^-$ and leads to the formation of the full spectra of AOS (*77–79*). Chinese hamster ovary (CHO) cells loaded with SOD exhibit decreased cytotoxicity when exposed to paraquat, implicating $\bullet O_2^-$ as the mediator of paraquat toxicity. Cell loading with catalase had no effect (78). Human gingival fibroblasts (*80*) exposed to paraquat *in vitro* exhibited increased total SOD and catalase activity, but GPX activity decreased. Rat, feline, and human cells of type II epithelial cell origin (*81*) also evidenced increased total SOD activity.

Murine fibroblasts (*82*) transfected with the human CuZnSOD were more resistant to paraquat cytotoxicity. However, the increased resistance correlated more closely with increased GPX activity found in the clones than with increased CuZnSOD activity. Mouse fibroblasts transfected with human MnSOD (in which no concurrent rise in any other antioxidant enzyme activity was detected), displayed increased resistance to the cytotoxic action of paraquat (*83*). Pretreatment of adult rats (*77*) with endotoxin and hyperoxic conditions, which increased antioxidant enzyme activity prolonged survival of the animals 3-fold when exposed to paraquat. These studies indicate that paraquat induces increased expression of antioxidant enzymes which may provide some protective benefit in the lung.

Cigarette Smoke

Cigarette smoke is a major public health hazard which exposes the respiratory tract to substantial oxidant stress. One puff from a cigarette contains approximately 10^{15} oxidant radicals in the combined gaseous and particulate states. Reactions within smoke also prolong half-lives for many radical species. Alveolar macrophages isolated from smokers and smoke exposed hamsters exhibit over 2-fold increased activities of total SOD and catalase (*84*), with no corresponding increases in GPX activity. Filtering smoke to remove the particulate fraction completely abolished increases in antioxidant enzyme activity in macrophages, implicating either the particulate phase as the causative factor in the observed activity increases or an interactive effect between the particulate and gaseous components. Smoke-exposed hamsters also showed increased survivability in hyperoxic conditions indicating that compensatory increases of antioxidant enzymes in smoking animals are protective (*84*).

Certain antioxidant enzymes are also increased in cigarette smokers. Red blood cells from smokers contain more glutathione and catalase than those from nonsmokers, but the same levels of GPX are found in both groups (*85*). Red blood cells from smokers also provide greater protection against cytotoxicity of hydrogen peroxide in cultured pulmonary endothelial cells in comparison to RBCs from nonsmokers. These results again suggest a

functional significance for elevations in antioxidant enzymes.

Bleomycin

Bleomycin, one of a battery of potent tumor killing chemicals produced by Streptomyces verticillis, plays an important role in cancer chemotherapy. Bleomycin complexes with iron (Fe^{II}) intracellularly, and in the presence of molecular oxygen, liberates hydroxyl radicals. When a reducing agent is present, Fe^{III} can replace Fe^{II} and be reduced to Fe^{II}, producing O via the Haber-Weiss reaction. A major side effect of bleomycin therapy is lipid peroxidation of cell membranes in the lung which partly induces the inflammation and pulmonary fibrosis often seen in patients receiving this chemotherapeutic agent (*86–88*).

Intratracheal administration of bleomycin in hamsters results in increased activity of GPX, catalase and SOD (*89,90*). These increases were observed from 7 to 21 days after administration. Rats infused with bleomycin intratracheally showed an initial decrease in whole lung total SOD, catalase and GPX activity at 2 days postexposure (*91*). However at later time points, SOD was increased at 4 and 7 days, catalase remained normal, and GPX was increased at 28 days postexposure. Intratracheal administration of bleomycin may also decrease certain phospholipid fractions of lung surfactant, and infusion of liposome-encapsulated SOD and catalase after administration of bleomycin partly ameliorates this reduction (*92*). Intraperitoneal injection of desferriosamine, an iron chelator, and dimethylsulfoxide, a scavenger of •OH, inhibited lung injury after intratracheal administration of bleomycin (*93*). Because injection of SOD or catalase had no effects, this study suggests •OH and iron are important in bleomycin-induced lung damage.

Summary

Abundant evidence implicates antioxidant enzymes as important components of lung defense after oxidative stress. Many of these enzymes appear to be regulated independently at the molecular level although coordinate increases in biosynthesis and activity levels of some enzymes are observed under certain situations. Undoubtedly, the balance between levels of enzyme induction and the magnitude or extent of oxidant injury to lung determines the outcome of biological responses which may include either adaptation to oxidative stress or lung injury. Endogenous baseline levels of antioxidant enzymes in various lung cell types may also be important in susceptibility to oxidant-induced cell damage as high levels of constitutive enzymes may preclude inducibility.

Although heterogeneous effects are observed in many situations, a common denominator of several stresses (hyperoxia, paraquat, mineral dusts) is increases in expression of MnSOD. Alleviation of paraquat (*83*) and asbestos-induced (*94*) cell damage after transfection of genes encoding MnSOD into cells *in vitro* suggest a causal relationship between induction of this enzyme and prevention of cell death. That increased expression of MnSOD can serve as a biomarker of chronic inflammation or lung disease or a biosensor of oxidant stress is suggested by recent work in this laboratory showing elevated steady-state mRNA levels of MnSOD in cells from bronchoalveolar lavage of rats exposed to asbestos by inhalation (manuscript in preparation). Increases in mRNA levels occur in a dosage-dependent fashion directly related to the airborne concentrations of dusts.

In addition to the well-recognized antioxidant enzymes discussed in this review, a number of other antioxidants exist in the lung both intracellularly and extracellularly. These include substances in epithelial cell lining fluid, cerruloplasmin, heme oxygenase, and glutathione. Recent data suggest that the relative importance of these antioxidant sources may vary with the type and degree of oxidant stress. For example, in human mesothelial cells (*95*) the glutathione redox cycle plays an important role in detoxifying low levels of oxidants, with catalase providing protection against severe oxidant stress.

The cloning of genes encoding antioxidant enzymes and the development of shuttle vectors enabling overexpression of these enzymes in cells *in vitro* are exciting recent findings with implications for gene therapy. These studies should also shed light on the relative importance of various antioxidant enzymes after selective oxidant stresses. Moreover, the development of synthetic scavengers of AOS and techniques for more effective targeting of cells of the lung will allow both preventive and therapeutic approaches to disease associated with exposure to environmental oxidants.

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